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Fibrinogen Longmont: A Dysfibrinogenemia That Causes Prolonged Clot-Based Test Results Only When Using an Optical Detection Method

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A new fibrinogen variant was discovered as a result of discrepancies found in routine laboratory screening. The patient, a healthy 37-year-old woman, had a mild bleeding history. Initial coagulation studies on the patient revealed a prolonged prothrombin time (PT) and a normal activated partial thromboplastin time (APTT). Further investigation on the patient and her mother demonstrated both had a PT with no end point using an optical detection method (ACL3000+) and a normal PT using an electromechanical detection method (ST4 Clot Detection System). The APTT for both the patient and her mother were essentially normal with both optical and mechanical detection methods. The patient and her mother also had markedly prolonged thrombin time and reptilase time results on the ACL3000+, but they were normal on the ST4. Coagulation test results on the patient's father were all normal. We believe the fibrinogen defect in this family may affect fibrin polymerization only enough to effect light scatter interpretation, while there is enough polymerization to increase plasma viscosity and yield an end point using an electromechanical analyzer. This report should alert pathologists and clinicians to possible discrepancies between mechanical and spectrophotometric clot testing methods. Am. J. Hematol. 63:149–155, 2000.

Key words: dysfibrinogen; fibrinogen; prothrombin time

INTRODUCTION

Fibrinogen, a 340,000 dalton glycoprotein composed of three pairs of nonidentical disulfide-bonded polypeptide chains, is synthesized in hepatocytes. Thrombin cleaves peptide fragments from the α and β chains of fibrinogen to form fibrin monomers that assemble into a polymeric fibrin clot which is essential for hemostasis [1,2].

Disorders involving fibrinogen have been classified as both quantitative deficiencies and qualitative abnormalities. However, careful examination has shown that most cases of hypofibrinogenemia also exhibit dysfunctional proteins. Thus, most fibrinogenopathies are now believed to be qualitative fibrinogen abnormalities and should be considered dysfibrinogenemias [3].

More than 200 cases of dysfibrinogenemias have been described in the medical literature. The most commonly observed functional fibrinogen defects are abnormal fibrinopeptide release and abnormal polymerization of fibrin monomers [3–5].

Although structural defects in the fibrinogen molecule may predispose to bleeding or thrombosis, the great majority of patients with fibrinogen abnormalities are asymptomatic. Many cases have been identified incidentally by abnormal routine coagulation test results. The most commonly observed laboratory test abnormalities are a prolonged thrombin time or reptilase time [4].

This paper reports an unusual case of a dysfibrinogenemia which was discovered by chance during a routine laboratory screening prior to surgery. The patient had a prothrombin time (PT) with no end point using an optical detection method while the PT was totally normal when

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a mechanical detection method was used. The patient's activated partial thromboplastin time (APTT) was essentially normal with both optical and mechanical detection methods.

CASE REPORT

A 37-year-old woman was evaluated for excessive vaginal bleeding which was suspected to be secondary to uterine fibroids. She was scheduled to have a hysterectomy when pre-operative clotting studies at Longmont United Hospital revealed a prolonged PT of >17 sec (normal range: 10.1–12.9 sec) and an APTT of 31 sec (normal range: 22–32 seconds). The patient was on no medications that could cause these laboratory findings.

Her past medical history was remarkable for frequent epistaxis until her mid-teens and intermittent excessive bruising. At age 19 the patient had excessive bleeding with the vaginal delivery of her only child which required the transfusion of six units of blood. She also has had gingival bleeding with tooth brushing but no excessive bleeding with dental extractions. The patient has no history of thrombosis. Her family history indicates that this disorder is genetic. Her mother has had massive hemorrhages following childbirth on three separate occasions. One of her three brothers has problems with chronic epistaxis, but her other two brothers appear normal. There was no family history of thrombosis.

The patient was referred to the University Hospital Special Coagulation laboratory for further hemostasis workup. Her mother and father were also available for study.

MATERIALS AND METHODS

All blood for coagulation testing was drawn into 3.2% buffered sodium citrate (vol/vol, 9 parts blood/1 part citrate) and centrifuged for 15 min at 4°C. The plasma was immediately separated, aliquoted, and either tested immediately or stored at −70°C prior to testing.

PT and APTT measurement at Longmont United Hospital were both performed on a MLA 700 (MLA, Pleasantville, NY) analyzer using an optical detection method and Dade (Miami, FL) reagents. APTT and PT testing at University Hospital were performed using both photometric clot detection on the ACL 3000+ (Instrumentation Laboratories, Lexington, MA) and electromechanical clot detection method on an ST4 coagulation analyzer (American Bioproducts, Parsippany, NJ). APTT and PT assays with both the photometric and electromechanical instrumentation utilized either the Organon Teknika (Durham, NC) automated APTT reagent or the Organon Teknika Excel S PT reagent. The reference ranges and coefficient of variation (CV) for these assays are as follows: ACL 3000+—PT, 11.2–14.3 sec, 1.5% CV; APTT, 24.4–35.4 sec, 2.6%CV; and ST4—PT, 13.3–15.9 sec, 2.9% CV; APTT, 26.4–40.1 sec, 2.3% CV.

The clot formation curves for both the PT and APTT were obtained using the research program on the ACL 3000+. These curves are a plot of relative light scatter as compared to the instrument standard versus time in seconds. Typically, the PT or APTT in seconds is determined from the clot curve using an algorithm programmed by the instrument manufacturer.

A manual tilt tube technique also was used to assay the PT on the patient and her parents using the Organon Teknika Excel S reagent according to instructions provided by the manufacturer.

Both activity and antigenic methods were used to determine the fibrinogen concentration. Fibrinogen activity was assayed using the Clauss method on the ST4 analyzer with Organon Teknika Fibriquik reagent according to manufacturer's instructions. Reference range and CV for fibrinogen activity are 170–400 mg/dL, 5.0% CV. Fibrinogen level by antigenic determination was measured using Laurell rocket [6] immunoelectrophoresis with a rabbit polyclonal antibody to human fibrinogen (Accurate Chemical and Scientific Corp., Westbury, NY). Reference range and CV are 170–400 mg/dL, 13.5% CV.

Additional clot-based testing on this patient and her mother included the thrombin time (TT), reptilase time (RT), and euglobulin lysis time. The TT was assayed on the ST4 coagulation analyzer using Dade Thrombin Clotting Reagent (Dade, Miami, FL) following the manufacturer's instructions. The TT was also assayed on the ACL3000+ using a 1:2 (vol/vol) dilution of the thrombin time reagent. Clot formation curves for the ACL3000+ TT were obtained using the research program. The RT was performed with Reptilase-R (Abbott Laboratories, Chicago, IL) on the ST4 analyzer according to the manufacturer's directions. The RT was also performed on the ACL3000+ using a 1:2 (vol/vol) dilution of the RT reagent. The ELT was performed manually according to a published procedure [7]. Reference ranges and CV for these assays are as follows: TT, 10.7–13.5 sec, 4.9% CV; RT, 11.0–14.0 sec, 3.5% CV; and ELT, 90–300 min, 6.0% CV.

Fibrinogen-crossed immunoelectrophoresis (CIE) was performed by electrophoresis of the patient plasma or a normal pool plasma through a 1% agarose gel (Seakem ME, FMC Bioproducts, Rockland, ME) and then pouring a second dimension perpendicular to the first. This second dimension consists of a 1% agarose gel containing a rabbit polyclonal antibody to human fibrinogen. The buffer used for this procedure was 0.03 M barbital, 0.016 M Tris pH 8.6, 0.25 M EDTA, and 0.01% sodium azide. After soaking in normal saline, drying the gel, and stain-

TABLE I. Coagulation Assays on the Patient and Her Parents

	Patient	Mother	Father	Reference ranges
PT (ACL 3000+)[a]	>150 sec	>150 sec	11.3 sec	10.9–14.7 sec
PT (MLA)[a]	>17 sec	Not done	Not done	10.1–12.9 sec
PT (ST4)[b]	13.0 sec	14.2 sec	13.2 sec	13.3–15.9 sec
PT (Manual/tilt tube)	13.4 sec	14.1 sec	12.5 sec	Control = 14.7 sec
APTT (ACL 3000+)[a]	31.4 sec	23.9 sec	31.2 sec	23.2–35.2 sec
APTT (MLA)[a]	31 sec	Not done	Not done	22–32 sec
APTT (ST4)[b]	33.4 sec	Not done	Not done	26.4–41.1 sec
Thrombin time (ACL 3000+)[a]	22.3 sec	22.0 sec	10.9 sec	Control = 11.0 sec
Thrombin time (ST4)[b]	13.5 sec	14.2 sec	10.8 sec	10.7–13.5 sec
Reptilase time (ACL 3000+)[a]	>150 sec	>150 sec	13.5 sec	Control = 13.3 sec
Reptilase time (ST4)[b]	13.8 sec	13.0 sec	13.8 sec	11–14 sec
Fibrinogen (Clauss method)	495 mg/dL	515 mg/dL	455 mg/dL	170–400 mg/dL
Fibrinogen antigen	418 mg/dL	445 mg/dL	484 mg/dL	170–400 mg/dL
ELT	668 min	518 min	328 min	90–300 min

[a]Optical clot detection instrument.
[b]Electromechanical clot detection instrument.

ing with Coomasie blue, a characteristic curve for fibrinogen was visualized. This characteristic curve, when compared to that of normal fibrinogen, can highlight qualitative protein defects.

Since the patient had a prolonged PT, all extrinsic factor activities were assayed. This testing was performed using a modified PT on a Coag-a-mate X-2 (Organon Teknika, Durham, NC) using the Organon Teknika Excel S PT reagent. All factor-deficient plasmas were either procured in-house or from a commercial source (George King Biomedical, Overland Park, KS). The CV for coagulation factor functional assays ranges from 5% to 10%.

Western blots were performed on plasmas from the patient, her parents, and normal pool. A plasmin digest of normal plasma and plasmas from the patient and her parents was performed as follows: 10 units/mL of human plasmin (Haematologic Technologies, White River Junction, VT) were added to the plasmas and incubated at 37°C for 2 hr. Both digested and undigested plasma samples were diluted 1:200 in saline and electrophoresed on a 10% SDS-PAGE, blotted to nitrocellulose, and stained for fibrinogen. The blot was developed using a polyclonal anti-human fibrinogen antibody (Dako, Inc., Carpinteria, CA) and VectaStain anti-rabbit IgG kit with alkaline phosphatase substrate kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Kaleidoscope prestained standards (Bio-Rad, Hercules, CA) were run in the leftmost lane of the gel.

RESULTS

Table I summarizes coagulation testing on the patient and her parents. Patient PT and APTT assays on both the ACL3000+ and the ST4 analyzers were performed three times on three different patient plasma samples drawn 1 and 12 months apart. Testing on the patient's parents was performed on a single venipuncture sample. On all occasions, the patient's APTT did reach an end point, whether tested using spectrophotometric or electromechanical clot detection. Only with the spectrophotometric clot detection method was the patient PT markedly abnormal. The electromechanical PT was within the reference range on all occasions. The patient's mother also demonstrated a markedly prolonged PT using an optical clot detection method and a normal PT with the electromechanical instrument. Both of the patient's parents had a normal APTT.

The manual/tilt-tube PT results reported in Table I were normal for the patient and her parents. S.C. described the clots formed in the samples from both the patient and her mother as "very pliable, semi-solid, and not holding their shape." The clots formed in the patient's father and control sample were described as "more rigid and maintaining the cylindrical shape of the surrounding test tube." Since we no longer routinely do manual PT's, a control value performed on pooled normal plasma has been reported in place of a reference range.

Results of the thrombin time and reptilase time (Table I) for the patient and her mother show a discrepancy between data derived from the optical and electromechanical analyzers. The thrombin times are prolonged when performed on the ACL3000+ and essentially normal using the ST4. Likewise, the reptilase times show a similar pattern. These same assay results for the patient's father are normal. Since the thrombin time and reptilase time are not routinely performed in this laboratory on the ACL3000+, a control assay performed on pooled normal plasma is listed in lieu of a reference range.

The euglobulin lysis time is a global test of the fibrinolytic system. The end point of this test is the dissolution of a fibrin clot formed from the patient's plasma. The patient and her mother have moderately prolonged ELT (Table I) results, while the patient's father shows a minimal prolongation. This prolongation in the father may represent normal test variation.

The patient and her parents all show somewhat increased levels of functional and antigenic fibrinogen (Table I). The functional or Clauss assay is a clot-based test using the ST4 electromechanical detection system. Both the functional and antigenic fibrinogen levels are concordant.

Because factors II, V, VII, and X can also affect the PT, they were assayed in the patient. All of these factor levels were found to be normal.

Figure 1 shows the clot formation curves of normal pool, patient, and her parents' plasma samples for both the APTT and PT assayed on the ACL3000+. The research program on the ACL 3000+ allows visualization of these curves. Basically, this instrument determines light scatter from the sample and also from the instrument's reference emulsion and plots a curve of sample light scatter/reference light scatter versus time. The ACL 3000+ uses cuvettes configured in a rotor. The rotor spins at 1,200 rpm, and consequently the machine measures light scatter on any sample at 1,200 times/minute. In Fig. 1 the PT and APTT plots labeled "pool plasma" are normal curves. These curves show a vertical segment representing the increase in light scatter during clot formation and a horizontal segment representing the maximum light scatter reached after the clot has fully formed. What the ACL 3000+ reports as time to clot formation is based upon an algorithm constructed by the manufacturer using the clot formation curve. For the patient PT and her mother's PT, there is a minimal increase in light scatter that the instrument does not detect. Because no increase in light scatter is measured, the ACL3000+ reports a PT greater than the maximum time the machine is programmed to report. For the APTT, a maximum light scatter is obtained for both the patient and her mother, although this increase appears much less than that for pool plasma. The patient's father shows essentially a normal clot formation curve.

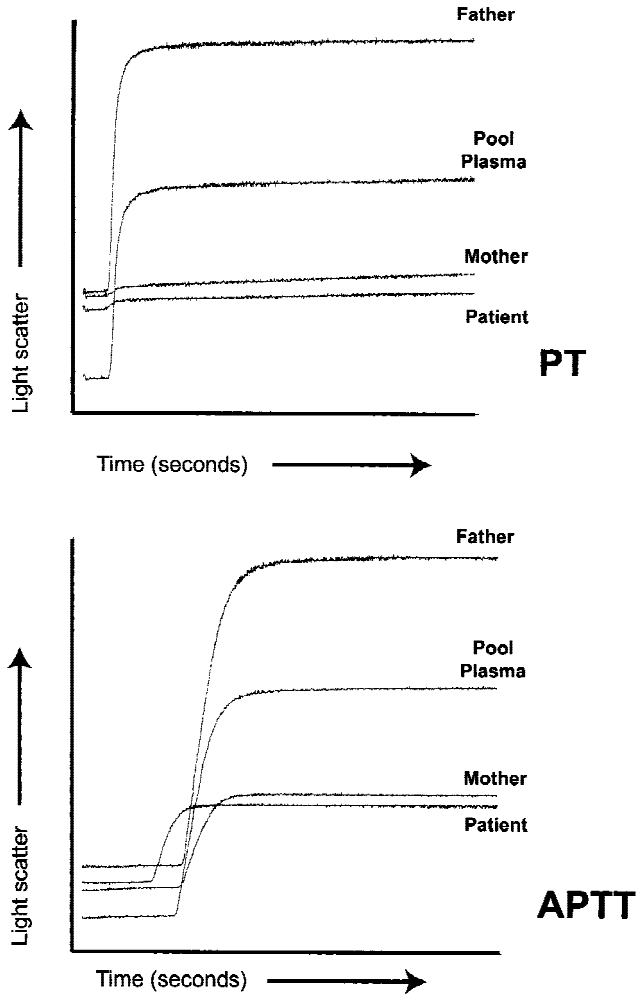

Fig. 1. PT and APTT clot formation curves from the ACL3000+ spectrophotometric instrument.

Figure 2 shows the clot formation curves for the thrombin and reptilase times performed on the ACL3000+. Again the patient and her mother show minimal increase in light scatter for both tests. In the case of the thrombin time, enough increase in light scatter occurs for the ACL3000+ to report a prolonged end point. The reptilase time shows no increase in light scatter for either the patient or her mother. The patient's father shows normal clot formation curves for both assays.

Figure 3 illustrates the fibrinogen CIE results from both a normal control and the patient. The patient's CIE curve is different from that of a normal control suggesting the presence of a qualitative fibrinogen abnormality.

Figure 4 shows a Western blot of native fibrinogen and that treated with plasmin. Native fibrinogen from the patient and her mother appears to migrate normally, producing a single band above the 201K molecular weight marker, as does fibrinogen from the father and pool plasma. The lower molecular weight bands of the digested fibrinogen from the patient and mother, compris-

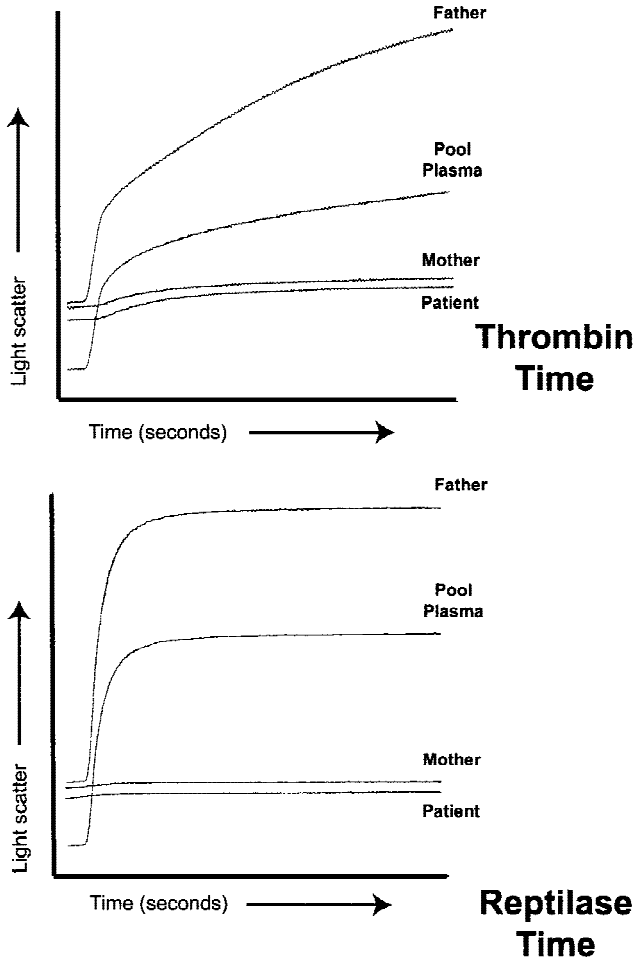

Fig. 2. Thrombin time and reptilase clot formation curves from the ACL3000+ spectrophotometric instrument.

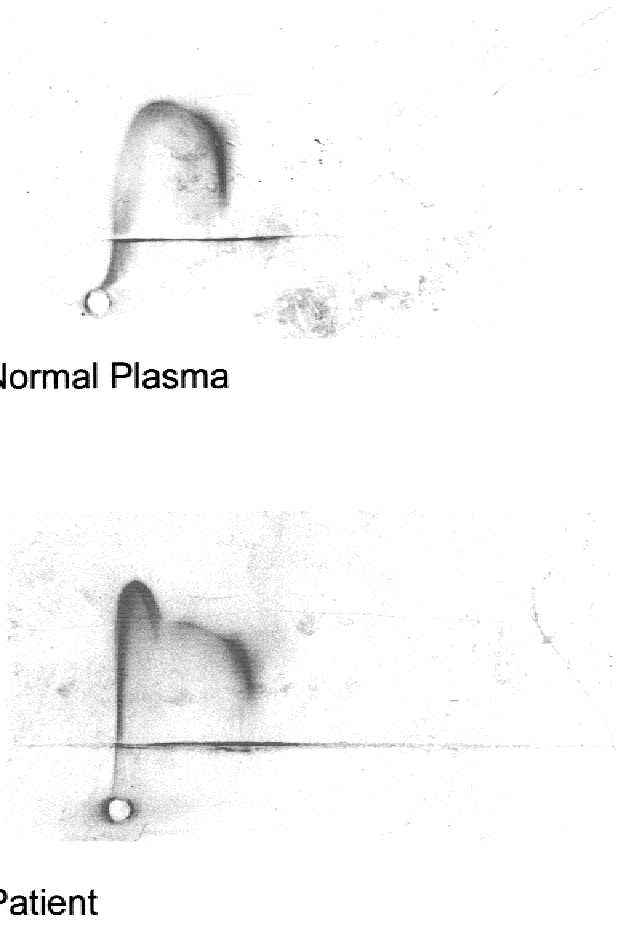

Fig. 3. Fibrinogen-crossed immunoelectrophoresis gels from a normal control plasma and the patient.

ing the bulk of the digest, also appear as bands identical to the controls. However, there are 3 bands between a molecular weight of 201K and 122K that appear in the patient's and the mother's digest which do not appear in the controls. While very faint bands in this general location are present in the controls, especially in the father's fibrinogen, they are not at the same molecular weights as those of the patient and her mother.

DISCUSSION

The formation of fibrin from fibrinogen can be divided into three phases: 1, proteolytic cleavage of fibrinogen by thrombin; 2, self-polymerization of fibrin monomers into fibrin polymer; and 3, chemical cross-linking of the fibrin polymer. There have been reports of abnormalities of all three phases of fibrin formation [8]. In general, of the hereditary dysfibrinogenemias that have been studied in detail, the primary defect appears to be alterations in amino acid sequence, usually single amino acid substitutions [4].

In this case, the patient was initially brought to our attention because of an abnormal prothrombin time noted on pre-operative laboratory studies. Further investigation at our institution revealed an abnormal spectrophotometric PT using the ACL 3000+, but a PT within the reference range using the mechanical ST4 analyzer. Studies on her parents demonstrated that her mother also had the same pattern of test results, indicating the defect was inherited from this parent. The functional defect in the fibrinogen is reflected by the abnormal clot formation curves obtained using the research program on the ACL 3000+ (Figs. 1 and 2). In addition, although the spectrophotometric APTT is normal, the clot formation curve also shows an attenuated increase in light scatter as compared with a normal control.

Other testing that lends further evidence to support the presence of a dysfibrinogen in this family include results obtained with the manual/tilt-tube PT, ELT, the plasmin digest, and fibrinogen CIE. The quality of the patient clot and that of her mother was described as "not holding its shape" by the technologist who performed the test. This suggests a qualitative abnormality in the clot structure. The prolonged ELT found in both the mother and patient

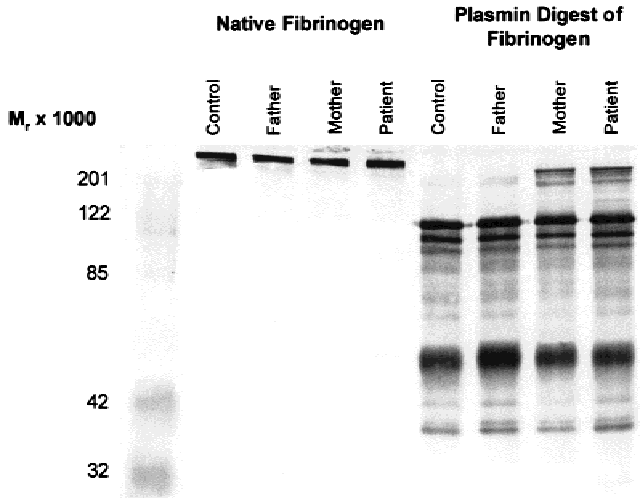

Fig. 4. Western blot of SDS-PAGE gel stained for human fibrinogen.

indicates some resistance to plasmin degradation of their clots. Dysfibrinogenemias with resistance to plasmin lysis have been reported to be associated with thrombotic symptoms [3]. However, there have been case reports indicating that this is not always so [9]. Our patient, to date, has not displayed any history of thrombosis. The plasmin digest of plasma from the patient and her mother demonstrate the presence of high molecular weight bands not visualized in samples from a normal control or her father. This data indicates the presence of a dysfibrinogen, which is proteolyzed differently by plasmin than normal fibrinogen. Finally, the abnormal fibrinogen CIE is most consistent with the presence of a qualitative fibrinogen defect.

Fibrinogen is partially proteolyzed to fibrin monomer by release of two peptides: fibrinopeptide A from the Aα fibrinogen chain and fibrinopeptide B from the Bβ fibrinogen chain. The fibrin monomers then undergo self-assembly or polymerization to form a fibrin clot. Reptilase is the snake venom batroxobin which causes the release of fibrinopeptide A from native fibrinogen [10]. Release of fibrinopeptide A is sufficient to cause clotting of the treated plasma sample. Defects in the release of fibrinopeptide A have been reported on multiple occasions to be a cause of dysfibrinogenemia and hence prolonged clotting tests. In this case, the reptilase time on the electromechanical instrument was normal for the patient and her parents but abnormal for the patient and her mother using a spectrophotometric analyzer. These results along with the clot formation curve (Fig. 2) suggest that the release of fibrinopeptide A is normal in this family. The defect appears to be abnormal fibrin monomer assembly that causes increased transparency in the clots of both the patient and her mother causing abnormal test results using a spectrophotometric analyzer.

We were unable to locate any previously published reports of clinical laboratory results showing major discrepancies between clinically utilized clot detection methods. Webb et al. [8] reported an abnormal fibrinogen, fibrinogen New Britain, in which the PT and PTT were both mildly prolonged, while the thrombin time and reptilase time were more severely prolonged. Furlan et al. [11] reported a functionally abnormal fibrinogen, fibrinogen Geneva, in which the APTT, thrombin time, and reptilase time were all prolonged, but the PT was shortened. Somewhat similar to the case reported here, spectrophotometric measurement of the fibrin monomer aggregation of fibrinogen Geneva showed an impaired increase in light scatter.

Formation of fibrin polymer is the end point detected in the major clotting time tests of the coagulation system. The ST4 clot detection system is based on the electromechanical detection of increasing viscosity of the plasma by utilizing the pendular swinging motion of an iron ball. An algorithm uses the variations in oscillation amplitude to determine the clotting time. The ACL instrument is based on centrifugal analysis using a channel to detect light scatter and, thus, polymerization of fibrin. All of the data from the patient and her family indicate the discrepancy between the mechanical and spectrophotometric test results are due to a defect which affects fibrin polymerization only enough to effect light scatter interpretation, although there is enough polymerization to increase plasma viscosity and yield an end point using the mechanical test.

In conclusion, we have reported a new fibrinogen variant which causes mild to moderate bleeding symptoms in the affected patient. The interesting element of this case is that the patient consistently displays a markedly prolonged spectrophotometric PT with a normal electromechanical PT. To our knowledge, this is the first case in the literature to report any abnormal fibrinogen with PT results of this nature. This report should alert pathologists and clinicians to possible discrepancies between mechanical and photometric PT testing methods. In accordance with accepted conventions, we have designated this fibrinogen variant fibrinogen Longmont.

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